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Activation of Aromatic Amines to Mutagens by Bovine Bladder and Liver Cells

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A bovine bladder cell-mediated mutagenesis system using Chinese hamster V79 cells and *Salmonella typhimurium* as target organisms was developed to investigate the capacity of the bladder urothelium to activate chemical carcinogens. Bovine bladder epithelial cells can activate the aromatic amines AF and 4-ABP to intermediates which mutate V79 cells and *S. typhimurium* TA 98 and TA 100. DMBA was mutagenic to V79 cells but not detectably mutagenic to either Salmonella strain with bladder cell activation. The chemicals tested were not mutagenic to either target organism in the absence of bladder cells. In contrast to the response with DMBA, *S. typhimurium* was a more sensitive target for the arylamines than V79 cells. These data suggest the value of using multiple end points for assessing metabolic capability. The activation capability of intact bladder cells was compared to disrupted cells, and S-9 prepared from bladder cells used with and without cofactors. When intact cells or S-9 plus cofactors were used as the activation system a dose-dependent increase in revertants was observed for 4-ABP. A bovine liver cell-mediated bacterial mutagenesis system was also developed and the liver and bladder systems compared. For AF, bladder cells appear to be at least ten times more active per viable cell than hepatocytes in producing mutagenic intermediates, while 4-ABP is essentially not mutagenic in the hepatocyte-mediated system. A quantitative comparison of the relative importance of the liver and bladder to activate the chemicals is difficult to make but the data indicate the ability of the bladder epithelium to activate bladder carcinogens.

Introduction

The role of specific chemicals associated with human neoplasms was first observed with arylamine-induced bladder cancer in workers associated with chemical dye and rubber industries (*1-5*). Many subsequent studies have shown that aromatic amines are carcinogenic in experimental animals and that species differences in the incidence of tumors exist. It is known that procarcinogens such as aromatic amines require metabolic activation to manifest their biological effects (*6, 7*). For the initiation of bladder cancer by aromatic amines two major theories concerning the organ site of activation have emerged. One theory relies on metabolic activation and conjugation occurring in the liver and the conjugated amine reaching the bladder via the urine (*8, 9*). The reactive intermediate which can interact with cellular constituents of the bladder epithelium is generated by hydrolysis of the conjugate in the urine. This hypothesis assumes a somewhat passive role for the bladder epithelium in carcinogen activation. An alternate theory on bladder tumor initiation is that metabolic activation of a carcinogen, or its metabolite, to ultimate reactive form(s) occurs enzymatically within the bladder epithelium itself. Indeed, studies have indicated that the bladder epithelium of several species, including humans, can metabolize carcinogens (*9-14*). Until recently little was known about the role of the urothelium during bladder tumor induction. Rapid absorption, metabolism, and re-excretion of arylamines after direct instillation into the bladder

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was observed by Oglesby et al. (*15*) and others (*16-18*). These studies suggest that the parent chemical and some metabolites are able to traverse the luminal urothelial membrane and that the urothelium is not as inert or impermeable to xenobiotics as earlier believed. The two theories on liver or bladder as sites of metabolic activation of bladder carcinogens may not be mutually exclusive as both organs may have significant roles with variables such as species and sex as well as chemical class of initiator being contributing factors.

We have been interested in developing *in vitro* cellular mutagenesis systems as a model to study the phenomenon of organ and species specificity of chemical carcinogens (*11, 12, 19-25*). The approach of cell-mediated mutagenesis was developed by Huberman and Sachs (*26*) and used Chinese hamster V79 cells as the mutable target and rodent embryonic fibroblasts as the activating cell. A hepatocyte-mediated mutagenesis system was developed (*19*) to expand the spectrum of chemicals which could be studied by this approach. Subsequently, rat fibroblast and rat hepatocyte activation of benzo(a)pyrene and aflatoxin B_1 were compared and a cell type specificity in carcinogen activation was demonstrated (*20*). We further extended this approach to include lung, kidney and bladder cells for metabolic activation which allowed an investigation of organ specificities of carcinogen activation (*22, 25*). A correlation between *in vitro* cell-mediated mutagenic activity for nitrosamines and hydrocarbons with *in vivo* carcinogenic activity for these classes of chemicals has been shown (*25*).

The data in Table 1 summarize some results comparing the relative abilities of rat liver and bladder cells to activate the carcinogens, dimethylnitrosamine (DMN) and 7,12-dimethylbenz(a)anthracene (DMBA) and the noncarcinogen, anthracene (*12*). As can be seen in Table 1, rat bladder epithelial cells can activate both of the carcinogens to mutagens, although the level of induced mutagenesis with DMN is less than with rat liver cell activation. DMBA is activated about equally in cells from both organs. These findings prompted us to investigate the activation of other chemical carcinogens by bladder epithelium and, furthermore, because of low rat bladder epithelial cell yield to utilize a species which would yield greater cell numbers. In the present report, data are presented for two aromatic amines, 2-aminofluorene (AF) and 4-aminobiphenyl (4ABP), which show (1) bovine bladder epithelial cells can activate aromatic amines to intermediates which mutate V79 cells and/or *Salmonella typhimurium*, (2) bovine bladder cells on a per cell basis give higher levels of *S. typhimurium* mutagenesis than bovine liver cells, and (3) bovine bladder urothelial cell S-9 can also activate AF and 4ABP to mutagens.

Table 1. Relative mutagenic activities of DMBA and DMN in the rate liver and bladder cell-mediated V79 mutagenesis system.

Chemical	Concn. μg/mL	Activating cell type[a]	
		Liver	Bladder
Anthracene	3	1	1
DMBA	1	14	21
DMN	100	95	11

[a]Data expressed as fold increases over background with ouabain as the selective agent in the liver cell-mediated system and 6-thioguanine as the selective agent in the bladder cell-mediated system. The ouabain background (liver) was 1 and 6TG background (bladder) was 3 for all experiments. (*12, 22*).

Materials and Methods

Carcinogen Preparation

Test chemicals were dissolved in dimethyl sulfoxide (DMSO) with solvent volume so adjusted that the final concentration of DMSO did not exceed 0.2% in V79 culture and did not exceed 100 μL per petri plate in the Salmonella tests. DMBA was purchased from Sigma Chemical Co. and AF and 4ABP were purchased from Aldrich.

Bladder Cell Isolation

The isolation of bovine bladder cells is described in detail elsewhere (*27*). Briefly, bovine bladders were obtained from a commercial slaughterhouse and kept on ice approximately 2 hr while being transported to our laboratory. Each bladder was filled with a trypsin-collagenase solution, suspended in a 500 mL beaker and incubated for 20-22 hr at 4°C. The tissue was then warmed to 37°C for 1 hr. To harvest the urothelial cells the enzyme was removed and the bladder lumen rinsed with sterile HBSS. The bladder was slit down the side, laid flat to expose the luminal surface and gently scraped with a sterile spatula to collect the loosened cells. The cell suspension was centrifuged at 300*g* for 5 min and resuspended in HBSS plus $CaCl_2$ and $NaHCO_3$ (*27*) for Salmonella mutagenicity tests or in complete medium (*27*) for V79 mutagenicity tests. An average of 100×10^6 viable cells (by trypan blue dye exclusion) per bladder was obtained.

Bladder Cell S-9 Mix Preparation

Ice cold bladder cell suspensions were sonicated with a probe microtip sonifier (Cell Disrupter Model 350, Branson Sonic Power Co., Danbury, CT) on a 50% on-off cycle for the least time required to break all cells, usually 20 to 30 sec (*28*). The broken cell suspension was centrifuged at 9000*g* at 4°C, for 20 min. The supernatant (S-9) was then mixed with

cofactors as suggested by Ames et al. (*29*) to prepare the S-9 mix. The S-9 equivalent of 4×10^6 intact cells (1.4 mg protein) was added to each plate. Protein was determined by the method of Lowry et al. (*30*).

Hepatocyte Isolation

Bovine liver cells were isolated by a modification (*28*) of the method of Fry et al. (*31*). Liver samples were obtained within 10 min of death (from the same slaughterhouse as bladders), were immediately cut in 2-cm cubes and were placed in ice cold PBS. About 3 g of 0.5 mm liver slices were rinsed three times in PBS for 10 min at 37°C in a shaking water bath and then two times in PBS with 0.5 *mM* EGTA. Following the rinses, 10 ml of sterile filtered collagenase-hyaluronidase solution was added to the liver slices and incubated for 45 min at 37°C. The partially digested tissue/cell suspension was filtered through a single layer of nylon screen and the undigested slices were returned to the flask and the collagenase-hyaluronidase digestion continued for three 30-min periods. Cells from the 30 min digestions were isolated, and their viability ranged from 80 to 90%.

Salmonella Mutagenesis Assays

Figure 1 shows a diagram of the cell-mediated bacterial system. The following were added to 2 ml of 45°C agar, 100 μL of a 16 hr nutrient broth culture of *Salmonella typhimurium* TA 100 or TA 98, the chemical, and 900 μL of the bladder cells, hepatocytes or S-9 mix. Two plates were prepared for each treatment. Revertant colonies were counted after 48 hr and background lawn of bacterial growth confirmed. Both the bladder cells and hepatocytes remained viable (by trypan blue dye exclusion) in the agar during the two day incubation.

V79 Cell Mutagenesis Assays

The V79 assay described by Langenbach et al. (*19*) was used with some modification (see Fig. 2). Bladder cells were added to 25 cm^2 T-flasks that had been seeded 18 hr earlier with V79 cells; two flasks per treatment were used. After a 4-hr cell attachment period, medium with floating cells was gently removed and fresh medium containing the chemical was added. Following a 48-hr chemical treatment period, the cells were trypsinized and counted on a hemacytometer. The first cloning

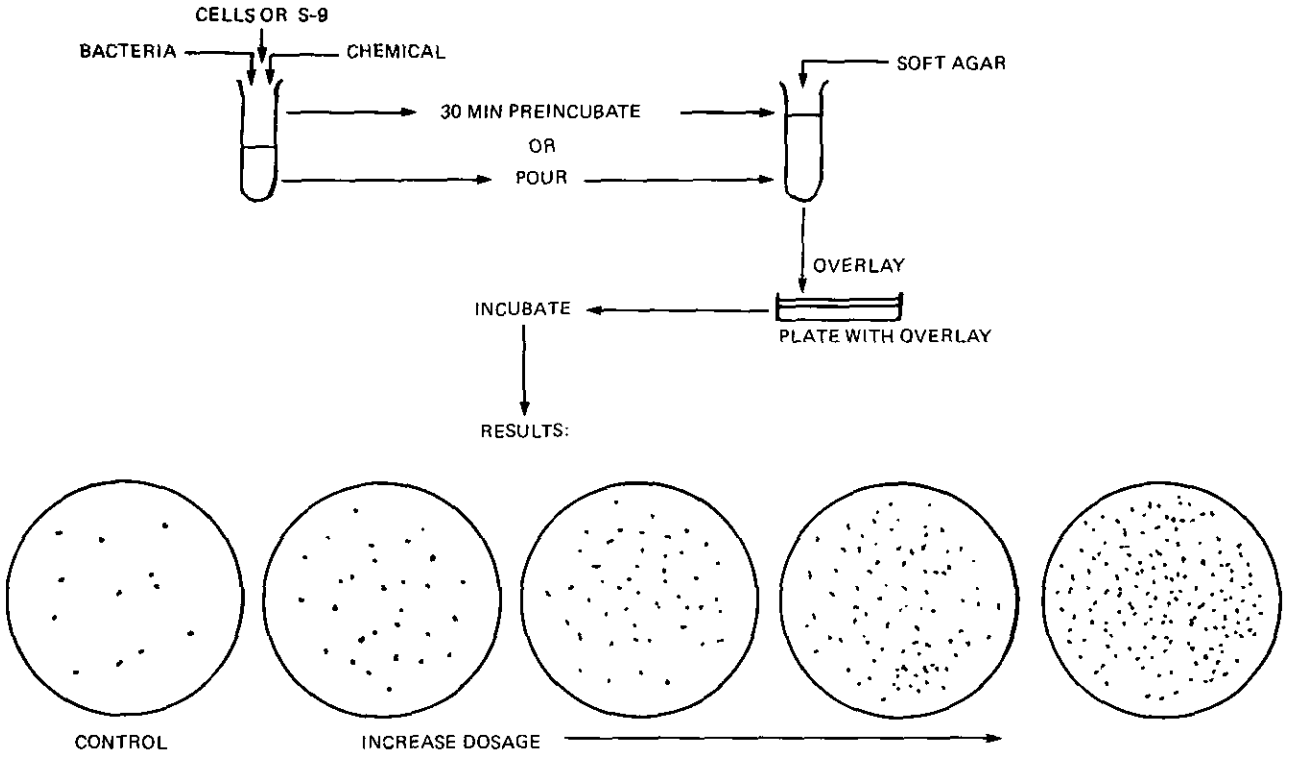

FIGURE 1. Bovine bladder cell-mediated *Salmonella typhimurium* mutagenesis system experimental protocol.

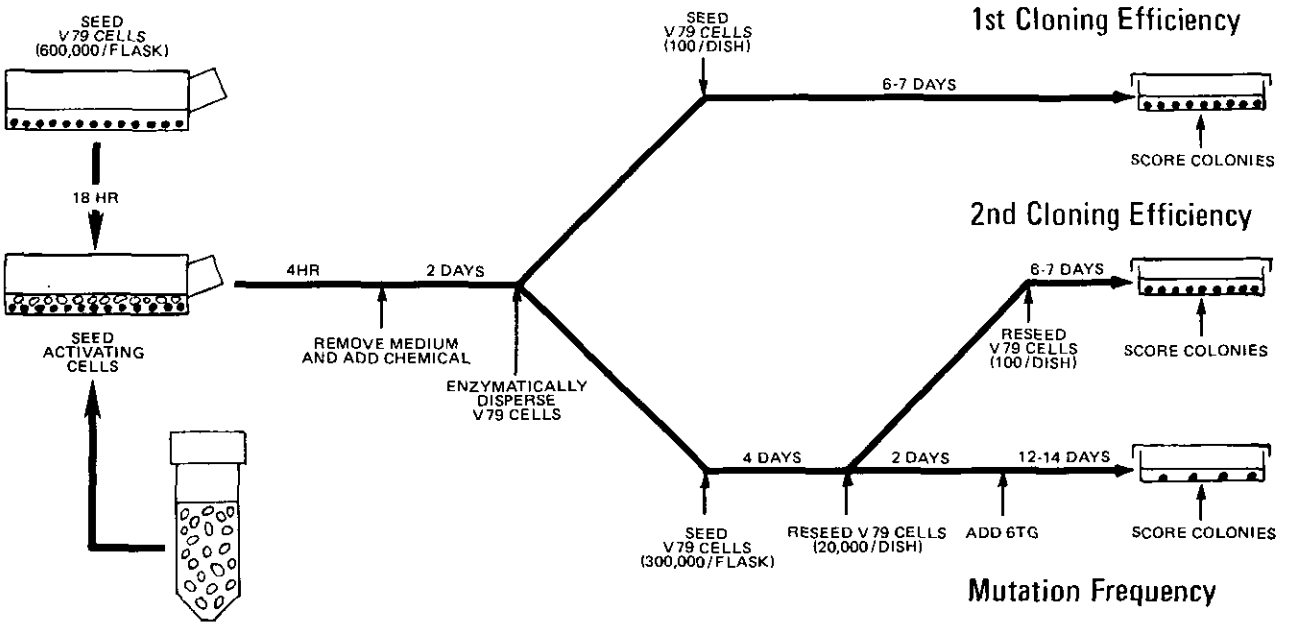

FIGURE 2. Bovine bladder cell-mediated V79 mutagenesis system experimental protocol.

Table 2. Bladder cells-mediated mutagenesis of V79 cells and *Salmonella typhimurium*.

	Bacterial mutagenesis			V79 mutagenesis		
Chemical	Concn, μg/plate	Revertants per plate[a] TA100	TA98	Concn, μg/mL	Cloning efficiency %[b]	No. 6-TG^r mutants /10^6 survivors
	Control	112	18	Control	83	12
AF	10	1890	2083	5	64	170
	20	2199	2382	10	50	217
4-ABP	10	911	148	10	68	87
	20	1381	291	20	64	15
DMBA	10	173	41	1	57	176

[a]To optimize mutagenesis, bacteria were incubated with the following number of bladder cells per plate: AF, 4ABP, DMBA 4 × 10^6.

[b]First cloning efficiency. To obtain optimum mutation frequencies. V79 cells were cocultivated with the following number of bladder cells: AF, 8 × 10^6; 4ABP, 15 × 10^6; DMBA, 4 × 10^6. These values are from representative experiments which have been repeated at least twice.

efficiency plates were seeded for a measure of toxicity (eight dishes/point, 100 cells/plate). Two 75 cm^2 T-flasks per treatment point were seeded with 3 × 10^5 V79 cells and allowed to grow for 4 days. The cells were then reseeded for cloning efficiency and 6-thioguanine resistance (2 × 10^4 cells/dish, 15 dishes/treatment point). The 6-thioguanine was added 2 days later to a final concentration of 5 μg/mL. The mutation frequency was calculated per 10^6 survivors based on the second cloning efficiency and the number of cells seeded for mutant selection.

Results

Bladder-Cell Mediated Mutagenesis

The data in Table 2 show mutagenic activity of the aromatic amines, AF and 4ABP, and the hydrocarbon, DMBA, in the bladder cell-mediated *S. typhimurium* and V79 cell systems. DMBA is included since it was previously shown to be positive in the rat bladder-mediated V79 cell system (Table 1). AF and 4ABP were mutagenic to TA 98 and TA 100 and to V79 cells with bovine bladder cell activation. DMBA was mutagenic to V79 cells but not detectably mutagenic to TA 98 and TA 100 with bovine bladder cell activation. None of the chemicals were mutagenic to either target organism in the absence of bladder cell activation. The number of bladder cells to give optimum mutagenic response for each chemical has been determined and were used to obtain the data in Table 2 (27). For the *S. typhimurium* system, the optimum cell numbers were the same for AF and 4ABP while DMBA was negative at bladder cell numbers up to 10 × 10^6 per plate. How-

ever, in the V79 system different bovine bladder cell numbers were optimal for each chemical. Linear dose responses at the optimum cell number ranged up to 10 and 20 μg/plate for AF and 4ABP, respectively, before toxic effects were observed in the *S. typhimurium* system (data not shown). Dose responses for AF in the V79 system were linear up to 20 μg/mL while for 4ABP, 10 μg/mL gave maximal mutagenic activity; with 5 μg/mL (data not shown) and 20 μg/mL the mutagenic response was lower. This may be due to a narrow dose-response range, although high toxicity was not observed in ranges where mutation decreased (Table 2). At higher doses, toxicity of the chemicals or metabolites to the bladder cells themselves may be the cause of decreased mutagenic activity. This complicated dose-response phenomena for 4ABP in V79 cells and greater sensitivity (fold increase over background) for both AF and 4ABP with the bacterial system caused us to use *S. typhimurium* as the target in the remaining studies with AF and 4ABP.

Hepatocyte and Bladder Cell Activation of Aromatic Amines

The relative abilities of bovine hepatocytes and bovine bladder cells to produce mutagenic intermediates from AF and 4ABP are shown in Table 3. The data have been expressed as revertants (minus background) per 6×10^6 viable activating cells to facilitate a comparison of the two cell types (*28*). For AF, the bladder cells appear to be at least ten times more active on a per cell basis than hepatocytes in producing mutagenic intermediates. While 4ABP was essentially not mutagenic in the hepatocyte-mediated system, it was mutagenic with bladder cell activation.

Comparison of Intact Bladder Cells and Bladder Cell S-9 Activation

The aromatic amines, 4ABP and AF have been studied for mutagenic activity to TA 98 with disrupted bladder cells or with the bladder cell S-9 equivalent with and without cofactors (Fig. 3). In these studies bladder cells and bladder S-9 were prepared from the same animal to eliminate inter-animal variation which can be as much as 10-fold (*27*). For 4ABP, both intact cells and S-9 show a dose dependent increase in revertants, but the mutagenic activity in the bladder S-9 was about three times greater at all doses than intact cells (Fig. 3.). However, if the bladder cells are disrupted and the homogenate without cofactors is used, the mutagenic activity remains at the background level. This indicates that leakage of enzymes is not responsible for the mutagenic activity observed with the intact cell system. Similar data are also shown for AF with intact cells and S-9 activation. However, for AF, bladder S-9 activation showed only a slight increase over the response with intact cells. As with 4ABP, incubation of AF with sonicated bladder cells without addition of cofactors, causes no increase in reverants per plate (data not shown).

Discussion

The results reported herein combined with other studies (*11, 12, 27, 28*) demonstrate that the bladder epithelium can metabolically activate several classes of chemical carcinogens, including aromatic amines, hydrocarbons, and nitrosoamines, to intermediates which mutate V79 cells and/or *S. typhimurium*. Uehlke (*32*) first demonstrated *N*-oxidization of aromatic amines by bladder epithelium from several species. However, Brill (*33*) using dog bladder mucosa observed low levels of *N*-oxidization of aromatic amines. More recently, Poupko et al. (*10*) demonstrated 4ABP *N*-hydroxylase activity in bovine bladder mucosal microsomes. Preliminary studies we have conducted suggest that dog bladder epithelial cells are less active than bovine bladder cells in activating aromatic amines, which is in agreement with the separate findings of Brill (*33*) and Poupko et al. (*10*). Such findings may indicate species differences in the metabolic capabilities and therefore resultant roles of the bladder epithelium in the activation of bladder carcinogens.

We found it interesting that bovine hepatocytes were less active on a per cell basis than bovine bladder cells in activating AF and 4ABP to mutagens. It should be stressed that mutagen production and release from the two cell types are being measured, and the data do not necessarily imply that total metabolism is following the same patterns. Indeed, different metabolic pathways may predominate in the

Table 3. Bovine hepatocyte and bladder cell activation of aromatic amines to mutagens in *Salmonella typhimurium*.[a]

Chemical	Concn, μg/plate	TA 98 revertants per plate[b]	
		Hepatocyte-mediated	Bladder cell-mediated
2-Aminofluorene	10	306	3098
	20	390	3546
4-Aminobiphenyl	10	9	195
	20	2	409

[a]To allow comparisons of equivalent activating cells, revertants were corrected to 100% viability and 6×10^6 cells per plate. Experiments required 4×10^6 bladder cells and 6×10^6 hepatocytes for these chemicals for optimum response.
[b]Spontaneous revertants subtracted; a average was 23.

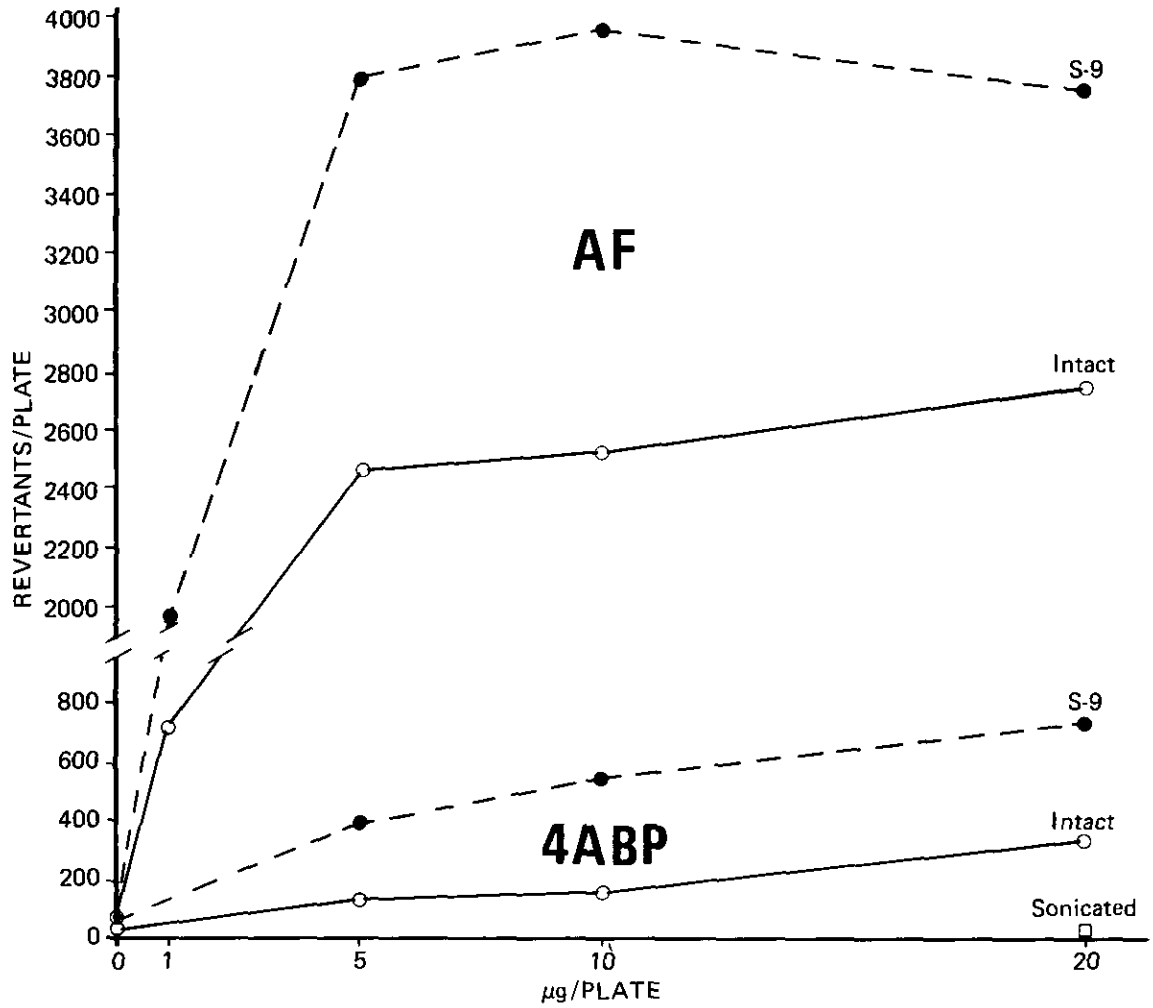

FIGURE 3. Comparison of activation of AF and 4ABP by intact bladder cells and cell fractions. 4 × 10^6 cells or S-9 equivalent in 900 μL was added per plate.

liver, where detoxification and conjugation may be major pathways with less release of mutagenic intermediates. On the other hand, the bladder may have less conjugation potential and therefore release reactive intermediates more readily. However, in agreement with our mutagenesis results, Poupko et al. (*10*), using *N*-hydroxylation of 4ABP as a measure of metabolism, observed substantial activity in bovine bladder microsomes with undetectable activity in bovine liver which further suggests a role for the bovine bladder in activating aromatic amines. Evidence that the bladder is not involved in aromatic amine activation comes mostly from carcinogen instillation and pellet implantation studies (*34*). In dogs and rats, such treatment with parent amines did not result in tumor formation, while treatment of dogs with the *N*-hydroxynaphthylamine did induce tumors (*34-36*). However, implantation with other carcinogens such as dibenzocarbazole (*37*), 3-methylcholanthrene and certain azo compounds have caused bladder tumors (*34*). Again, species variation in response may account for some of these differences and general conclusions about the lack of involvement of bladder epithelium activation may be premature. Furthermore, while the liver may be an initial site for aromatic amine metabolism, the actual time and concentration the chemical is in contact with the liver could be variable, whereas the bladder could encounter concentrated levels of the chemical and/or metabolites for prolonged times. Also, there is a large difference in cell numbers between the two tissues and even with less activating capability per cell, total mass would probably allow the liver to metabolize greater levels of chemical. How such factors could influence the relative importance of liver and

bladder carcinogen activation in the initiation of bladder cancer is unknown.

The need for cautious choice of the target organism is also indicated in the present studies. With bladder cell activation, V79 cells detected DMBA, AF and with a lower sensitivity, 4ABP as mutagenic. Conversely, *S. typhimurium* with bladder cell activation was not reverted by DMBA, but detected AF and 4ABP as mutagenic with significant increases over background. At present the causes of the different sensitivities of the target organisms can only be speculated about, but the data indicate the need for using multiple endpoints when assessing activation potential.

Bovine bladder S-9 was also capable of activating aromatic amines. In fact, the equivalent bladder S-9 was more effective in activating AF and 4ABP to mutagens for *S. typhimurium* than intact bladder cells (Fig. 3). This increased activity with S-9 could be due to trapping of active intermediates in intact cells, loss of conjugating enzymes upon cell disruption, closer proximity of S-9 enzymes to the target organism and/or other causes. However, it should be emphasized that the chemical class as well as target organism can determine which activation system is optimal as nitrosamines and hydrocarbons are sensitively detected with intact cellular activation and V79 cells as the target (*25*). Furthermore, intact cells are advantageous, since enzyme systems other than those requiring NADPH may be contributing to metabolic activation of some bladder carcinogens. Prostaglandin synthetase is an example of such an enzyme system which may be involved in carcinogen activation in the bladder (*38*).

In summary, the present results indicate the presence of enyzmes in bovine bladder cells which activate aromatic amines to mutagens, and suggest a possible role of the bladder epithelium in carcinogen activation.

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